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# Confinement of Therapeutic Enzymes in Selectively Permeable Polymer Vesicles by Polymerization-Induced Self-Assembly (PISA) Reduces Antibody Binding and Proteolytic Susceptibility

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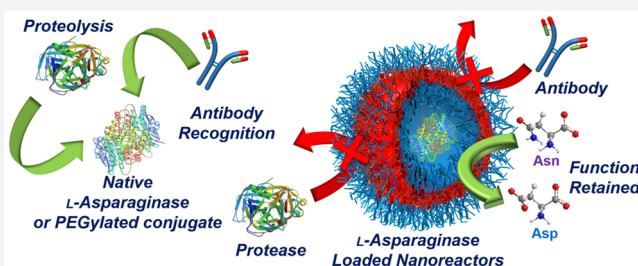
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## Supporting Information

**ABSTRACT:** Covalent PEGylation of biologics has been widely employed to reduce immunogenicity, while improving stability and half-life *in vivo*. This approach requires covalent protein modification, creating a new entity. An alternative approach is stabilization by encapsulation into polymersomes; however this typically requires multiple steps, and the segregation requires the vesicles to be permeable to retain function. Herein, we demonstrate the one-pot synthesis of therapeutic enzyme-loaded vesicles with size-selective permeability using polymerization-induced self-assembly (PISA) enabling the encapsulated enzyme to function from within a confined domain. This strategy increased the proteolytic stability and reduced antibody recognition compared to the free protein or a PEGylated conjugate, thereby reducing potential dose frequency and the risk of immune response. Finally, the efficacy of encapsulated L-asparaginase (clinically used for leukemia treatment) against a cancer line was demonstrated, and its biodistribution and circulation behavior *in vivo* was compared to the free enzyme, highlighting this methodology as an attractive alternative to the covalent PEGylation of enzymes.



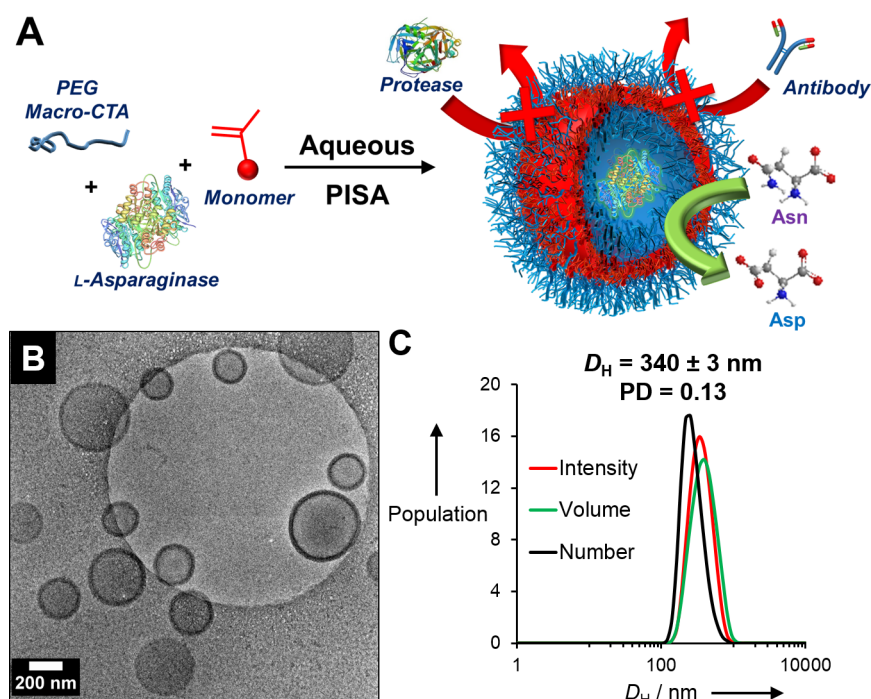
## INTRODUCTION

The clinical use of biologics (protein therapeutics) is the fastest growing pharmaceutical sector, with the global market forecast of \$380 billion by 2019.<sup>1</sup> The specificity of such drugs makes them ideal candidates for the targeted treatment of diseases. They are, however, susceptible to proteolysis and can induce an immune response if identified by lymphocytes. Poly(ethylene glycol) (PEG) is a non-immunogenic polymer commonly conjugated to proteins (PEGylation) to increase its hydrodynamic volume, reducing glomerular filtration and improving biological half-life.<sup>2,3</sup> Conjugation also reduces the proteolytic susceptibility and immunogenicity of the protein, improving its pharmacokinetics.<sup>4</sup> L-Asparaginase (ASNS) is a biologic sold under the trade name Kidrolase as an injectable treatment for acute lymphoblastic leukemia, by depleting external L-asparagine and hence preventing tumor growth. Numerous side-effects are associated with ASNS, driven by production of anti-ASNS antibodies,<sup>5</sup> which limits doses, thereby reducing the event-free survival rate.<sup>6</sup> These effects have been improved by

PEGylation (Oncaspar),<sup>7</sup> yet hypersensitive patients previously treated with ASNS still show an immune response to Oncaspar making switching treatments unviable.<sup>8</sup> Moreover, in patients who do not show hypersensitivity with Oncaspar, the biologic exhibits reduced efficacy upon anti-ASNS binding.<sup>8</sup>

Covalent PEGylation of proteins requires the chemical modification of residues, often in a nonspecific manner, which alters the protein's hydrophobicity and surface charge.<sup>9</sup> In contrast, encapsulation of unmodified proteins inside compartmentalized domains requires no residue modification, provides a physical shield against proteases, and also helps evade both innate and adaptive (antibody) immune responses.<sup>10–13</sup> However, for an encapsulated enzyme-therapeutic to exert its effect the substrates/products must permeate into the compartment necessitating multistep procedures to produce pores or other mechanisms of small-molecule sieving, often addressed by

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**Figure 1.** (A) Schematic of the ASNS-loaded vesicle preparation by aqueous polymerization-induced self-assembly (PISA) highlighting that the semipermeable membrane is hypothesized to act as a size-selective barrier allowing for therapeutic function, but not degradation or antibody binding. (B) Representative cryo-TEM image and (C) DLS size distribution of purified ASNS-loaded vesicles.

using speciality monomers<sup>14,15</sup> or post-synthetic procedures,<sup>16</sup> stimuli-responsive membranes,<sup>17–20</sup> membrane proteins,<sup>21–23</sup> or DNA nanopores<sup>24,25</sup> to impart permeability.

Herein, aqueous polymerization-induced self-assembly (PISA)<sup>26,27</sup> was utilized to encapsulate a clinical biologic, ASNS, inside inherently size-selectively permeable vesicles in order to protect it from external proteases and from antibody recognition (Figure 1A). After encapsulation, the enzyme remained catalytically active, demonstrating the membrane's permeability toward small molecules. The binding of ASNS antibodies was shown to be greatly reduced relative to both the native enzyme and the PEGylated conjugate. Furthermore, the encapsulated protein's stability to proteolytic degradation was shown to be higher *in vitro* and *in vivo*, when compared to the free enzyme. The efficacy of the ASNS-loaded vesicles toward a cancer cell line with down-regulated L-asparagine synthetase was also confirmed. Our approach is proposed as a possible alternative to PEGylation of therapeutic enzymes, as it may offer improved stability and reduced immunogenicity. As no chemical modification is required, this approach could be utilized for the encapsulation of a range of therapeutic enzymes, independently of their structure.

## RESULTS AND DISCUSSION

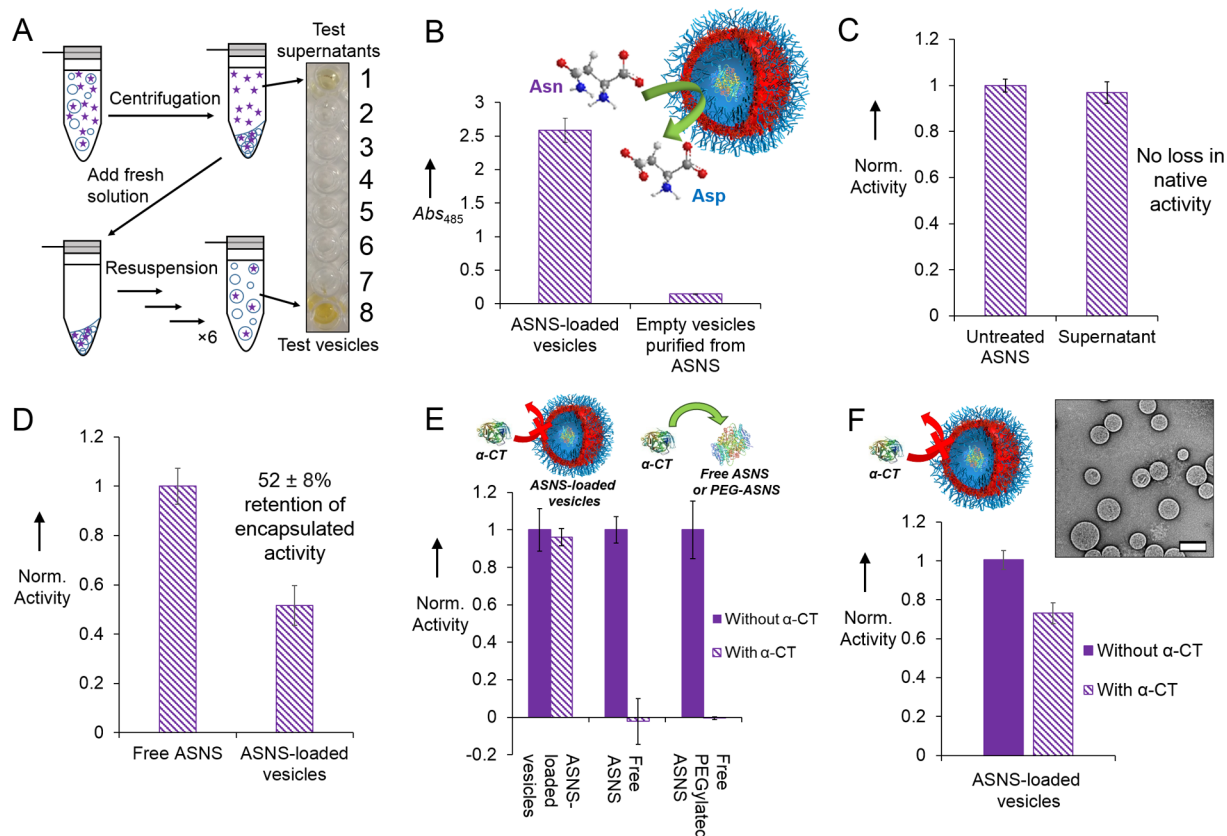
ASNS's mechanism of action is the breakdown of L-asparagine, a nonessential amino acid for healthy cells, into L-aspartic acid and ammonia. Certain cancerous cells, such as leukemia cells, underexpress the synthetase required for the synthesis of L-asparagine and are therefore starved of this amino acid in the presence of ASNS, in contrast to healthy cells, which synthesize their own L-asparagine.

Therefore, we hypothesized that semipermeable ASNS-loaded nanoreactors, which could allow diffusion of the substrate into the lumen, serve as an attractive alternative to PEGylation. Aqueous photo-PISA in the presence of the

protein yielded ASNS-loaded vesicles comprising a PEG shell and a poly(2-hydroxypropyl methacrylate) (PHPMA) membrane.<sup>27–30</sup> Such membranes are highly hydrated,<sup>31</sup> which enables size-selective transport of small molecules while protecting the protein from external macromolecules.<sup>30</sup> The ASNS-loaded vesicles were fully characterized by dynamic light scattering (DLS), atomic force microscopy (AFM), and dry-state and cryogenic transmission electron microscopy (TEM) after the excess protein was removed by repeated centrifugation/resuspension cycles (Figure 1B,C and Figure S1). Analysis of the supernatants from the purification confirmed complete removal of free ASNS (Figure 2A). The ASNS-loaded vesicles retained activity, whereas empty vesicles purified from an identical ASNS external solution showed no L-asparaginase-associated activity (Figure 2B) confirming no residual adhered protein on the polymersome surface or free protein in solution. Analysis of the ASNS in the first supernatant revealed no loss in activity after PISA relative to fresh enzyme, indicating that the photo-PISA conditions did not damage the protein (Figure 2C). The loading efficiency of the protein was determined by Western blot analysis of disassembled vesicles, which revealed a loading efficiency of 9% (Figure S2). Comparing the activity of the ASNS-loaded vesicles to the free protein showed retention of  $52 \pm 8\%$  activity (Figure 2D). The reduction in activity could be mainly ascribed to the diffusive barrier imparted by the semipermeable vesicle membrane, which retards the passage of substrate, as relevant control experiments showed no loss of activity under polymerization conditions with or without light (Figure S5).

The colloidal stability of empty and ASNS-loaded vesicles in a range of media (including cell culture media) was assessed over time, upon incubation at 37 °C (Figure S3). As expected, the hydrodynamic diameter of both blank and ASNS-loaded vesicles remained constant in deionized water (DI) over extended incubation periods. For both vesicles, a minor size



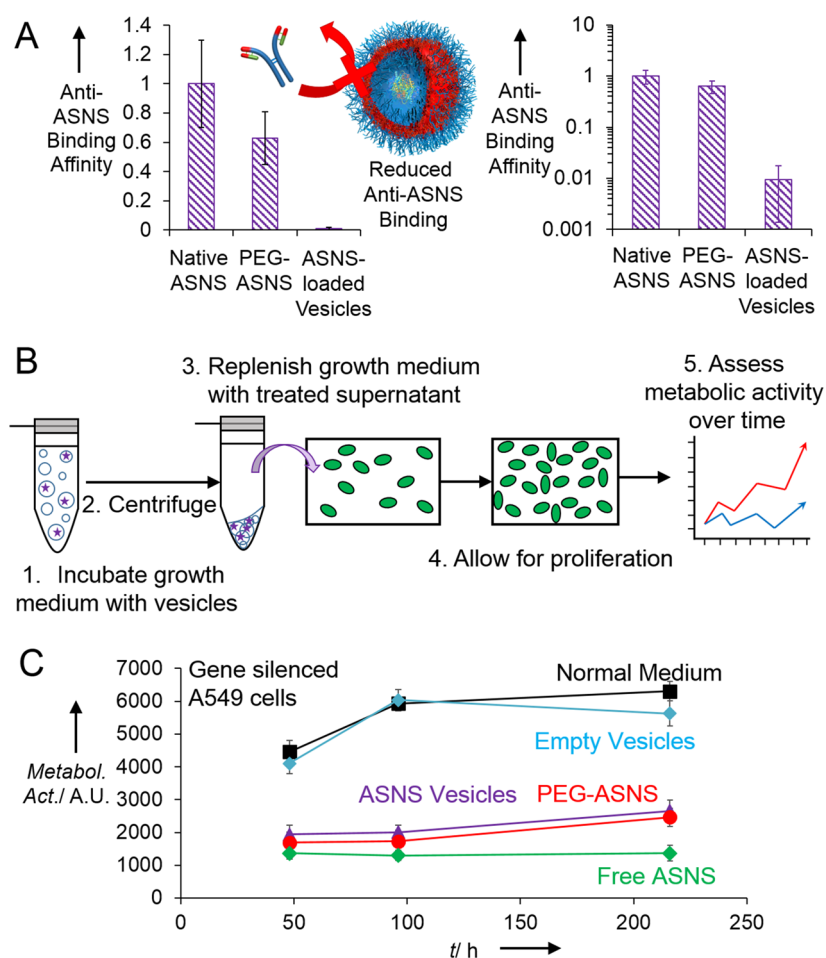


**Figure 2.** (A) Purification process by repeated centrifugation/resuspension cycles (end point microwells are shown) and (B) activity of ASNS-loaded vesicles compared to empty vesicles purified from ASNS. (C) Activity of free ASNS from supernatant after photo-PISA against untreated enzyme. (D) Activity of ASNS-loaded vesicles against free enzyme. (E) Proteolytic stability of ASNS-loaded vesicles, free ASNS, and free PEGylated ASNS after 18 h and (F) ASNS-loaded vesicles after 7 days. The inset shows dry-state stained TEM micrograph of vesicles after 7 days with  $\alpha$ -CT, scale bar = 500 nm. The normalized activity is defined as the ratio between the absorbance of the sample and the absorbance of free, untreated ASNS, at the end point of the enzymatic assay (end point = 30 min,  $\lambda$  = 485 nm).

increase was observed after 24 h upon incubation in fetal bovine serum (FBS) solution, suggesting slow agglomeration with blood proteins, while a slight decrease in size was monitored upon incubation of the vesicles in cell growth medium. These effects were more pronounced after 72 h; however, the empty and ASNS-loaded vesicles still maintained their overall size in the range 230–420 nm. These results demonstrate that both empty and ASNS-loaded vesicles have good colloidal stability in physiological media, for an extended time period. The ASNS-loaded vesicles also showed excellent proteolytic stability in the presence of a protease,  $\alpha$ -chymotrypsin ( $\alpha$ -CT), with quantitative retention of activity after 18 h exposure. Conversely, free ASNS and PEG-ASNS both completely lost their activity after this time (Figure 2E). Indeed, the ASNS-loaded vesicles remained intact after 7 days incubation with  $\alpha$ -CT, and the encapsulated ASNS remained active even after this time (Figure 2F). Based on these positive results, we hypothesized that antibodies, with larger molar masses than  $\alpha$ -CT, would also be prohibited from entering into the lumen, thereby preventing antibody recognition. Anti-ASNS binding was assessed using a sandwich enzyme-linked immunosorbent assay (ELISA).<sup>32</sup> The binding affinities of anti-ASNS toward both PEG-ASNS and the ASNS-loaded vesicles were compared to the binding toward a dilution series of native ASNS. This demonstrated the promiscuity of the binding toward the native ASNS or toward the PEG-ASNS conjugate,<sup>8</sup> which bound with affinities within an order of magnitude of

one another (Figure 3A). However, ASNS-loaded vesicles showed a binding affinity 2 orders of magnitude lower than the native protein. Therefore, unlike with PEG-ASNS treatment, treatment of hypersensitive patients with encapsulated ASNS could lower the risk of an immune response. As with any PEGylation strategy, PEG sensitization is still an issue (Figure S4); however non-linear PEG stabilizers show potential to reduce anti-PEG binding,<sup>32,33</sup> and indeed could be incorporated into such nanoreactors.

The empty and ASNS-loaded vesicles' cytotoxicity was assessed *in vitro* on A549 cells (human lung cancer fibroblasts). Cell viability was found to be  $\geq 90\%$  after incubating cells for 7 days with vesicle concentrations up to 2 mg mL<sup>-1</sup>, demonstrating low cytotoxicity (Figure S6). Furthermore, the ability of the ASNS-loaded vesicles to inhibit cell proliferation on ASNS gene silenced A549 was assessed *in vitro*. To generate cells deficient in L-asparagine synthetase (simulating leukemia), A549 (lung adenocarcinoma) cells were gene silenced with the appropriate siRNA making them dependent on external L-asparagine to proliferate. Western blot analysis of lysed cells treated with the siRNA revealed a 65% knockdown efficiency (Figure S7). Growth media was treated with either free ASNS, PEG-ASNS, empty vesicles, or ASNS-loaded vesicles, to deplete external L-asparagine. Treated media were then used to grow gene silenced cells, and metabolic activity was assessed at various time points (Figure 3B). As shown in Figure 3C, such cells showed a comparable reduction in proliferation when



**Figure 3.** (A) Anti-ASNS binding affinity toward native ASNS, PEG-ASNS, and ASNS-loaded vesicles shown on linear and logarithmic scales. (B) Schematic representation of the *in vitro* assay followed for the assessment of metabolic activity of ASNS gene silenced A549 cells over time. (C) Metabolic activity of ASNS gene silenced A549 cells over time grown in different treated media.

treated with the ASNS-loaded vesicles (58%), PEG-ASNS (61%), or free ASNS (78%), demonstrating similar enzymatic function. Cells treated with a growth medium incubated with the empty vesicles proliferated at a similar rate as the control, demonstrating the retention of cell function. In order to understand the vesicles' site of action, internalization of GFP-loaded vesicles<sup>30</sup> in A549 cells was investigated. After 12 h of incubation no internalization was observed (Figure S8). This supports an extracellular mechanism of action that the polymersomes do not need to enter cells to exert their therapeutic effect, in agreement with the mechanism of action reported for free ASNS.<sup>34</sup> Finally, the biodistribution and circulation behavior of ASNS encapsulated in our polymeric vesicles was compared to the free enzyme in an immunocompetent Balb/c mouse model. At 24 h post-injection, the free enzyme is seen to be present at significantly higher levels in both the liver and the kidneys compared to that encapsulated in the vesicles, indicating that the vesicles provide enhanced clearance protection of the enzyme (Figure S9). The accumulation of the vesicles at 48 h within the different clearance organs (spleen, liver, kidneys) is typical for nanoparticle systems.<sup>35–37</sup> This data implies a greater degree of immune-avoidance through the use of the “stealthy” vesicle structure, and shows great potential for the ASNS vesicles to be used as therapeutic delivery vehicles.

## CONCLUSIONS

A mild, one-pot methodology to stabilize protein therapeutics within permeable nanoparticles has been developed using PISA as an alternative to PEGylation for therapeutic enzymes. Our approach yielded inherently size-selective vesicles using commercial reagents at high solids content (11 wt %) in short reaction times under mild photoinitiated polymerization. The PHPMA membrane exhibits size-selective permeability, which allowed the therapeutic L-asparaginase to function by catalyzing the removal of asparagine from an external solution while protected inside the vesicle. The encapsulated protein exhibited a greater proteolytic stability *in vitro* and *in vivo* than the native protein or a PEGylated conjugate, while the immunogenicity of the encapsulated species was greatly reduced due to its location inside the polymersome. This approach does not chemically alter the protein of interest and can be applied to a wide range of therapeutic and functional proteins, and hence future research includes the encapsulation of a range of biologics, and further *in vivo* investigations.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00168.

Materials and characterization techniques, experimental details, additional characterization data of ASNS-loaded vesicles, loading efficiency and activity calculations, colloidal stability, cytotoxicity and cell internalization results, and *in vivo* biodistribution data (PDF)

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### Notes

The authors declare no competing financial interest.

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